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Effect of nitric oxide modulation on TGF-β1 and matrix proteins in chronic cyclosporine nephrotoxicity

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Background. Chronic cyclosporine (CsA) nephrotoxicity is characterized by interstitial fibrosis and afferent arteriolar hyalinosis. L-arginine (L-Arg), the substrate for nitric oxide (NO) synthase and N-nitro-L-arginine-methyl ester (L-NAME), the NO synthase inhibitor, were shown to modulate acute CsA nephrotoxicity. However, the mechanism of fibrosis in chronic CsA nephrotoxicity remains unclear. Thus, we examined the effect of NO modulation on fibrosis and the expression of transforming growth factor-β1 (TGF-β1) and matrix proteins in chronic CsA nephrotoxicity.

Methods. Rats were administered CsA (7.5 mg/kg), CsA + L-Arg (1.7 g/kg), CsA + L-NAME (3.5 mg/kg), vehicle (VH), VH + L-Arg, and VH + L-NAME, and were sacrificed at 7 or 28 days. NO production, physiologic parameters, and histology were studied in addition to the mRNA expression of TGF-β1, plasminogen activator inhibitor-1 (PAI-1) and the matrix proteins biglycan and collagens type I and IV by Northern and the protein expression of PAI-1 and fibronectin by enzyme-linked immunosorbent assay.

Results. While L-NAME strikingly reduced NO biosynthesis and worsened the glomerular filtration rate and CsA-induced fibrosis, L-Arg had the opposite beneficial effect. In addition, the CsA-induced up-regulated expression of TGF-β1, PAI-1, and the matrix proteins biglycan, fibronectin, and collagen I was significantly increased with L-NAME and strikingly improved with L-Arg. Collagen IV expression was not affected. Also, NO modulation did not affect VH-treated rats.

Conclusions. Chronic CsA nephrotoxicity can be aggravated by NO blockade and ameliorated by NO enhancement, suggesting that NO maintains a protective function. NO modulation was associated with a change in TGF-β1 expression, which, in turn, was associated with alterations in matrix deposition and matrix degradation through its effect on PAI-1.

The major dose-limiting adverse effect of long-term cyclosporine (CsA) administration is chronic nephrotoxicity [1, 2]. Chronic CsA nephrotoxicity may progress to an irreversible renal lesion characterized by striped tubulointerstitial fibrosis and hyalinosis of the afferent arterioles [3]. While acute CsA nephrotoxicity is thought to result from intrarenal vasoconstriction, the mechanism leading to the fibrosis of chronic CsA nephrotoxicity remains unclear. In order to study chronic CsA nephrotoxicity, a reproducible animal model with physiologic and histologic features in the kidney that resemble the human lesion described in patients on long-term CsA therapy was developed [4]. Our previous studies using the same model have shown that transforming growth factor-β1 (TGF-β1), a key fibrogenic cytokine implicated in the fibrosis of a number of chronic diseases, is involved in the fibrosis of chronic CsA nephrotoxicity by increasing matrix protein synthesis and by decreasing matrix degradation through increasing the activity of plasminogen activator inhibitor-1 (PAI-1) [5, 6]. We have also shown that the renin-angiotensin system (RAS) is up-regulated in this model and that blocking the RAS was beneficial in reducing fibrosis and the expression of TGF-β1 and various matrix components [7].

In the kidney, nitric oxide (NO) is a vasoactive factor important in maintaining vascular tone [8]. NO is produced from L-arginine (L-Arg) by the action of NO synthase (NOS) isoforms. The three NOS isoforms are present in the kidney [9]. Neuronal NOS (NOS-I) exhibits a macula densa cell specific expression. Inducible NOS (NOS-II) was observed in mesangial and proximal tubular cells, and endothelial NOS (NOS-III) is expressed mainly in endothelial cells of afferent and efferent arterioles and in glomerular capillaries. While NOS-I and NOS-III are constitutively expressed, NOS-II is normally undetectable in the kidney and is induced by cytokines [10]. Dietary L-Arg supplementation was shown to have beneficial effects in a number of experimental renal diseases presumably because it increases NO production [11–14]. In contrast, N-nitro-L-arginine-methyl ester (L-NAME), a potent competitive inhibitor of NO synthesis, was shown to worsen kidney disease. In the kidney, NO is not

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only involved in glomerular hemodynamics regulation, water-electrolyte balance, and renin production but was also shown to prevent glomerular thrombosis and ischemia [14, 15], lessen mesangial cell proliferation, decrease extracellular matrix protein synthesis [16], and decrease interstitial macrophage infiltration [17].

Several in vivo and in vitro studies have suggested that alterations of the NO pathway may be involved in the renal vasoactive responses and functional impairment seen with experimental acute CsA administration [18–24]. NO causes a relaxation of preglomerular arteries, thus improving renal blood flow. The endothelial-dependent vasodilation mediated by NO is impaired during acute CsA treatment and can be enhanced by simultaneous NOS inhibition with L-NAME, resulting in reductions in the glomerular filtration rate (GFR) [25]. Recently, dietary L-Arg supplementation was shown to be protective in chronic CsA nephrotoxicity [26]. However, only the histologic and physiologic changes were assessed, and the mechanism leading to this beneficial effect was not studied.

Thus, we tested the hypothesis that if CsA administration alters intrarenal NO synthesis, then potentially chronic NO blockade could worsen and chronic exogenous L-Arg administration could limit renal dysfunction and, more importantly, could have a beneficial effect on the fibrosis and the expression of TGF-β1 and extracellular matrix proteins in the setting of chronic CsA nephrotoxicity. Conditions of chronic NO blockade were established with L-NAME and chronic NO enhancement with L-Arg supplementation. Our study results support the hypothesis that NO maintains a protective function in chronic CsA nephrotoxicity.

METHODS

Experimental design

Adult male Sprague-Dawley rats (Charles River, Wilmington, MA, USA) weighing 200 to 250 g at the beginning of the study were used. They were housed in individual cages in a temperature- and light-controlled environment and received a low-salt diet (0.05% sodium; Teklad Premier, WI, USA), with water ad libitum. Animals were pair fed and were weighed and examined daily. After 7 or 28 days on the low-salt diet, weight-matched pairs of rats were randomized into six groups of six animals for a total of 12 experimental groups. Four groups were started on L-Arg (Sigma Chemical, St. Louis, MO, USA) at a dose of 1.7 g/kg/day added in powder form to a low-salt diet. After five days of L-Arg supplementation, CsA (Sandoz Research Institute, East Hanover, NJ, USA) at a dose of 7.5 mg/kg/d was administered subcutaneously to two groups, while two separate groups received a weight-based identical volume of vehicle (VH) olive oil as control. The CsA or VH was given concurrently with L-Arg and continued for 7 or 28 days. Four additional groups received L-NAME (Sigma Chemical Co.) and were treated with VH (two groups) or CsA (two groups) at 7.5 mg/kg/day for 7 or 28 days. L-NAME was dissolved in distilled water at a concentration of 5 mg/100 mL. The rats were allowed free access to L-NAME solution in standard drinking bottles. The average daily intake for the rats was 23 mL (3.5 mg of L-NAME per kg). The doses were chosen based on previous experiments done in the same model [25, 26].

The experimental groups of rats were categorized as follows: group 1, CsA (7 days); group 2, CsA + L-NAME (7 days); group 3, CsA + L-Arg (7 days); group 4, VH (7 days); group 5, VH + L-NAME (7 days), group 6, VH + L-Arg (7 days); group 7, CsA (28 days); group 8, CsA + L-NAME (28 days); group 9, CsA + L-Arg (28 days); group 10, VH (28 days); group 11, VH + L-NAME (28 days); and group 12, VH + L-Arg (28 days).

After each treatment period, systolic blood pressure was measured by tail plethysmography (Natsume Seisakusyo, Tokyo, Japan), and 24-hour urine samples were collected in metabolic cages (Nalge, Rochester, NY, USA) into a plastic tube cooled with ice. The following day, rats were anesthetized with intraperitoneal ketamine. The abdomen was opened through a midline incision, and the aorta was cannulated retrogradely below the renal arteries with an 18-gauge needle. With the aorta occluded by ligation above the renal arteries and the renal veins opened by a small incision for outflow, the kidneys were perfused with 20 mL of cold heparinized saline. The left kidney was removed and processed for light microscopy and protein analysis. After removing the right kidney, the cortex was dissected from the medulla, and the whole cortex was processed for RNA analysis. After the experiment, the animals were euthanized by deep anesthesia with ketamine followed by exsanguination because of transection of a major blood vessel.

Functional studies

Blood was collected from the jugular vein in plastic syringes transferred to metal-free tubes and chilled on ice. Plasma was harvested immediately by centrifugation at 4°C and stored at −70°C until determined. Urinary and plasma creatinine were measured by a Cobas autoanalyzer (Roche Diagnostics, Div. Hoffman-La Roche Inc., Nutley, NJ, USA). Creatinine clearance (C_{Cr}) was calculated using a standard formula. CsA blood level was measured by a monoclonal radioimmunoassay for CsA (Incstar Co., Stillwater, MN, USA). Urinary excretion of the stable NO metabolites NO_2 and NO_3 was determined. Urinary NO_2 was assayed by the Griess reagent [27]. Urinary NO_3 was estimated by reduction to NO_2 using nitrate reductase from *Aspergillus* species (Boehringer Mannheim Biochemicals, Indianapolis, IN,

USA). The NO_3 reduction was 95 to 100% effective. Urinary NO_3 excretion was considered the difference between total NO_2 (after reduction) minus initial NO_2 values. The results are expressed in μmol per 24 hours of urine.

Morphology

Tissue samples were fixed in 10% buffered formalin and were embedded in paraffin. Sections 2 to 4 μ thick were stained with periodic acid-Schiff's reagent and trichrome stain. Histologic findings were subdivided into interstitial scarring and arteriolopathy. Tubulointerstitial fibrosis consisted of matrix expansion with tubular distortion and collapse and basement membranes thickening. Features of interstitial inflammation included mononuclear infiltrates, edema, and interstitial cells vacuolization. Renal arteriolopathy was characterized by eosinophilic granular transformation of afferent arterioles and progressed to hyalinization and destruction of the afferent arterioles and terminal portions of interlobular arteries. A minimum of 20 fields at ×100 magnification were assessed and graded in each biopsy by an observer masked to treatment groups using a color image analyzer (Mustek Pentagon 800 SP, MacIntosh Power PC 7100, NIH Image vs. 1.5).

Tubulointerstitial fibrosis was estimated by counting the percentage of injured areas per field of cortex and medulla and scored semiquantitatively as: 0 = normal interstitium, 0.5 = <5% of areas injured, 1 = 5 to 15%, 1.5 = 16 to 25%, 2 = 26 to 35%, 2.5 = 36 to 45%, and 3 = >45%. Hyalinosis of the afferent arterioles was semiquantitatively assessed by counting the percentage of juxtaglomerular afferent arterioles available for examination with a minimum of 100 glomeruli per biopsy assessed: 0 = no arterioles injured, 0.5 = <15%, 1 = 15 to 30%, 1.5 = 31 to 45%, 2 = 46 to 60%, 2.5 = 61 to 75%, and 3 = >75%.

Northern blot analysis

Renal tissue was finely minced with a razor blade on ice then homogenized in TRIzol reagent (GIBCO BRL, Grand Island, NY, USA). RNA extraction was performed according to the manufacturer's protocol. After resuspension in Tris-ethylenediaminetetraacetic acid (EDTA) buffer, RNA concentrations were determined using spectrophotometric readings at $Absorbance_{260}$. Thirty micrograms of RNA were electrophoresed in each lane in 0.9% agarose gels containing 2.2 mol/L formaldehyde and 0.2 mol/L Mops (pH 7.0) and were transferred to a nylon membrane (ICN Biomedicals, Costa Mesa, CA, USA) overnight by capillary blotting. Nucleic acids were crosslinked by ultraviolet irradiation (Stratagene, La Jolla, CA, USA). The membranes were prehybridized for two hours at 42°C with 50% formamide, 10% Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), 5 × standard saline citrate (SSC), and 200 μg/mL denatured salmon sperm DNA. They were then hybridized at 42°C for 18 hours with cDNA probes labeled with ^{32}P-dCTP by random oligonucleotide priming (Boehringer Mannheim). The blots were washed in 2 × SSC, 0.1% SDS at room temperature for 15 minutes and in 0.1 × SSC, 0.1% SDS at 50°C for 15 minutes. Films were exposed at −70°C for different time periods to ensure linearity of densitometric values and exposure time. Autoradiographs were scanned on an imaging densitometer (GS-700; Bio-Rad Laboratories, Hercules, CA, USA). The density of bands for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used to control for differences in the total amount of RNA loaded onto each gel line. For quantitative purposes, values were divided by the density of bands for GAPDH in the same lane.

The cDNA probes were a mouse TGF-β1 cDNA probe (plasmid MUI5) kindly provided by R. Derynck [28], a rat PAI-1 cDNA probe [plasmid pBluescript SK(−)] obtained from T.D. Gelehrter [29], a human biglycan cDNA probe (plasmid P16) from L.W. Fisher [30], a rat procollagen α1 cDNA (plasmid pa1R1) obtained from D. Rowe [31], a rat collagen IV cDNA probe (plasmid pCIV-1-PE16) purchased from American Type Culture Collection (Rockville, MD, USA) [32], and a rat GAPDH cDNA probe (plasmid pBluescript KS II), a gift from J.M. Blanchard [33].

Immunoassay

Frozen (−70°C) kidney tissue sections embedded in Tissue-Tek O.C.T. compound (Miles Inc., Diagnostics Div., Elkhart, IN, USA) were processed for protein extraction. Tissue was thawed at 4°C in 5 mL lysis buffer (GITC/BME) and then rinsed in 5 mL cold 4°C phosphate-buffered saline (PBS), pH 7.4. Protein extracts were prepared by homogenizing kidney tissue in 1 mL cold PBS containing 0.05% Tween 20 (PBS-T) with a glass tissue homogenizer (Kontes Glass, Vineland, NJ, USA). The homogenate was centrifuged at 4°C for 15 minutes at 15,000 × *g*, and the supernatant was collected and then centrifuged to remove cellular debris. Protein concentration in the supernatant was determined using the Micro BCA (bicinchoninic acid) assay (Pierce, Rockford, IL, USA).

To determine PAI-1 and fibronectin, an inhibitory enzyme-linked immunosorbent assay (ELISA) technique modified from the method of Rennard et al was used [34]. Wells in a microtiter plate were coated with rat PAI-1 antiserum (American Diagnostics, Greenwich, CT, USA) or goat antirabbit fibronectin (Pierce). To another plate, serial dilutions of standard or unknown samples and rabbit IgG anti-PAI-1 (American Diagnostics) or antifibronectin (American Diagnostics) were added. After washing the coated plate, aliquots of the second plate were added to the coated plate and in-

Table 1. Changes in creatinine clearance, urinary nitric oxide (NO) excretion, systolic blood pressure, and cyclosporine A (CsA) whole blood level in the experimental groups at 7 and 28 days

	Creatinine clearance *mL/min/100 g*	Urinary NO *μmol/24 h*	Blood pressure *mm Hg*	CsA level *ng/mL*
7 Days				
CsA	0.33 ± 0.03	7.8 ± 1.0	130 ± 4	1279 ± 207
CsA + L-NAME	0.27 ± 0.02[a]	5.0 ± 0.7[a]	136 ± 3	1331 ± 65
CsA + L-Arg	0.35 ± 0.01	12.2 ± 1.4[a,b]	132 ± 5	1348 ± 124
VH	0.42 ± 0.01	7.9 ± 1.0	121 ± 4	0
VH + L-NAME	0.37 ± 0.02	5.3 ± 1.0	137 ± 3	0
VH + L-Arg	0.46 ± 0.03	12.9 ± 1.2[a]	138 ± 4	0
28 Days				
CsA	0.26 ± 0.02[a]	6.9 ± 1.2	125 ± 6	2422 ± 214
CsA + L-NAME	0.16 ± 0.00[a,b]	4.1 ± 0.7[a,b]	124 ± 33	1923 ± 498
CsA + L-Arg	0.38 ± 0.06[a,b]	13.6 ± 2.3[a,b]	127 ± 2	2014 ± 56
VH	0.41 ± 0.02	8.5 ± 1.2	129 ± 5	0
VH + L-NAME	0.43 ± 0.02	5.4 ± 0.9[a]	139 ± 7	0
VH + L-Arg	0.44 ± 0.04	13.1 ± 1.9[a]	133 ± 2	0

Data are mean ± SEM of six animals.
Abbreviations are: CsA, cyclosporine; L-NAME, N-nitro-L-arginine-methyl ester; L-Arg, L-arginine; VH, placebo.
[a] $P < 0.05$ vs. VH
[b] $P < 0.05$ vs. CsA

cubated. After washing, horseradish peroxidase-conjugated goat antirabbit IgG (Pierce) was added and developed using standard methods. Absorbance (450 nm) was read using a microtiter plate reader (Molecular Devices, Palo Alto, CA, USA).

Statistical analysis

Results are presented as mean ± SE. Comparisons between groups were done by analysis of variance (Kruskal–Wallis test, followed by Tukey test). The level of statistical significance was chosen as $P < 0.05$.

RESULTS

Physiologic studies

Values for GFR, urinary excretion of NO, systolic blood pressure, and CsA whole blood trough levels are summarized in Table 1. Weight gain was progressive in all of the treatment groups (data not shown). There were no significant differences in body weight except in the CsA + L-NAME group at 28 days, which failed to gain as much weight (312 ± 42 g, $P < 0.05$ vs. CsA, 393 ± 7 g). Whole blood trough CsA levels progressively increased but remained comparable among the CsA-treated groups. As expected, CsA treatment significantly decreased GFR compared with the VH groups. L-NAME administration significantly worsened GFR in the CsA-treated group, with the difference achieving statistical significance ($P < 0.05$) at 28 days. On the other hand, L-Arg treatment protected animals treated with CsA from impaired GFR but did not return the values to the VH-treated groups level. This effect was seen only in the CsA-treated groups since NO manipulation with L-NAME and L-Arg did not change GFR in the VH-treated groups.

L-NAME administration significantly decreased urinary NO excretion by 36 to 41% and 33 to 36% in the CsA and VH groups, respectively. On the other hand, urinary excretion of NO was significantly increased with L-Arg treatment by 56 to 97% and 54 to 63%, respectively, in the CsA and VH groups. While CsA treatment lowered urinary NO excretion compared with the VH group, the difference did not achieve statistical significance. There were no significant differences in systolic blood pressure among all of the treatment groups. L-Arg treatment did not lower the blood pressure in either the CsA or the VH groups.

Table 2. Semiquantitative scoring of tubulointerstitial fibrosis and arteriolopathy in the different groups at 7 and 28 days

	Tubulointerstitial fibrosis		Arteriolopathy	
	7 Days	28 Days	7 Days	28 Days
CsA	0.1 ± 0.1	2.1 ± 0.3[a]	0.1 ± 0.1	1.5 ± 0.7[a]
CsA + L-NAME	0.2 ± 0.1	2.8 ± 0.3[a]	0.3 ± 0.2	1.8 ± 0.6[a]
CsA + L-Arg	0.0 ± 0.0	0.9 ± 0.2[b]	0.1 ± 0.1	1.0 ± 0.2[b]
VH	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1
VH + L-NAME	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1
VH + L-Arg	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1

Data are mean ± SEM of six animals.
Abbreviations are: CsA, cyclosporine; L-NAME, N-nitro-L-arginine-methyl ester; L-Arg, L-arginine; VH, placebo.
[a] $P < 0.05$ vs. VH
[b] $P < 0.05$ vs. CsA

Histologic changes

The histologic changes observed are summarized in Table 2. The VH rats treated with L-Arg or L-NAME demonstrated normal kidney histology (Fig. 1A). By contrast, the kidneys of the CsA groups had characteristic morphologic changes mostly evident at 28 days. CsA-treated rats developed a striped pattern of tubulointerstitial fibrosis and tubular atrophy (Fig. 1B). While L-NAME

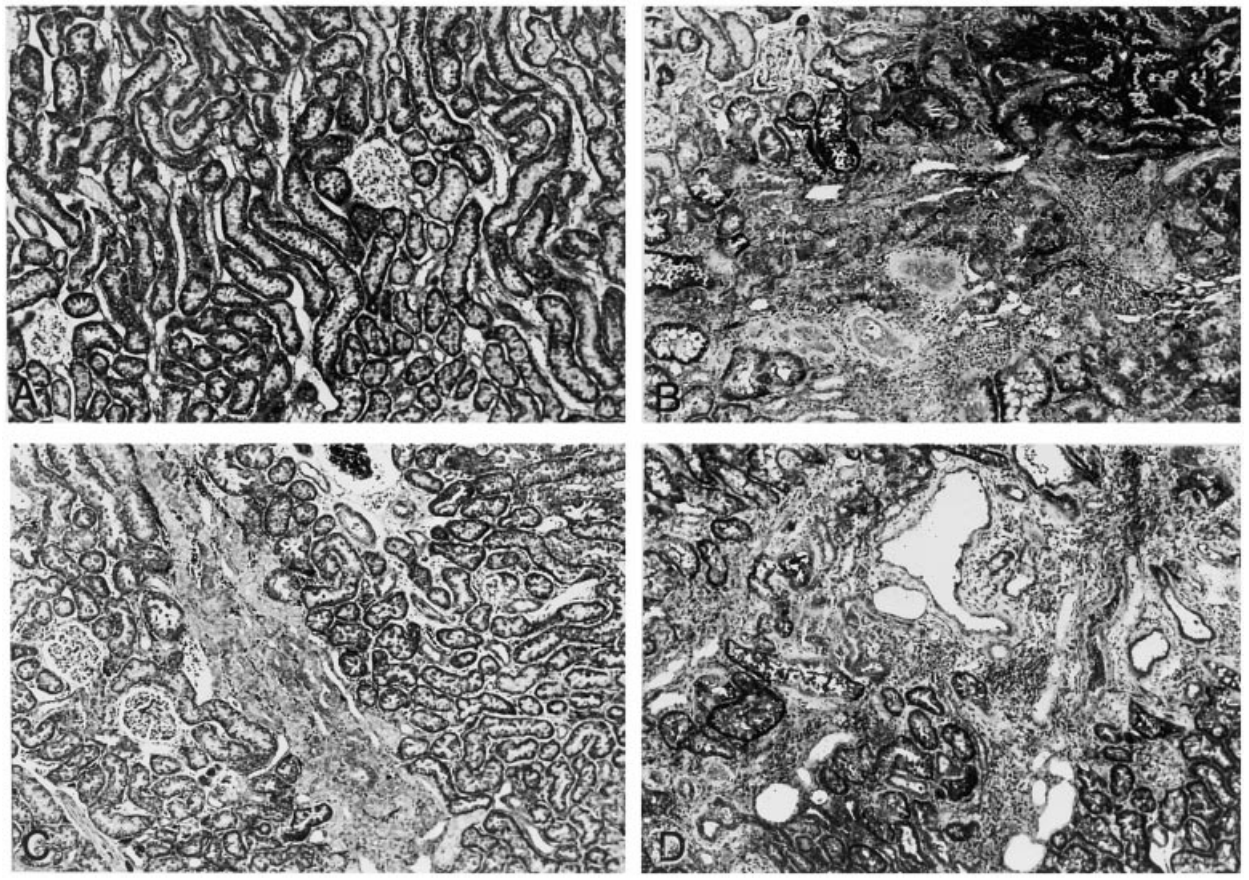

Fig. 1. Histologic changes in experimental chronic cyclosporine (CsA) nephrotoxicity. Photomicrographs showing the renal cortex of a salt-depleted rat given olive oil (VH, vehicle; *A*), CsA at 7.5 mg/kg/day (*B*), a combination of CsA at 7.5 mg/kg/day and L-arginine (L-Arg) at 1.7 g/kg/day (*C*), or a combination of CsA at 7.5 mg/kg/day and N-nitro-L-arginine-methyl ester (L-NAME) at 3.5 mg/kg/day (*D*) for 28 days. In rats treated with only CsA, interstitial fibrosis and tubular atrophy are seen relative to a medullary ray. When both CsA and L-Arg are given in combination, the interstitial fibrosis and tubular atrophy are less severe. In contrast, when both CsA and L-NAME are given in combination, the interstitial fibrosis and tubular atrophy are much more extensive (trichrome, magnification ×100).

treatment worsened the tubulointerstitial changes (Fig. 1D) at 28 days in the CsA group, L-Arg treatment resulted in significantly ($P < 0.05$) less severe tubulointerstitial fibrosis and tubular atrophy (Fig. 1C) compared with the CsA-only treated group. The kidneys of CsA-treated rats also showed prominent vascular lesions characterized by hypertrophied smooth muscle cells of the afferent arterioles with characteristic granular eosinophilic transformation. These arteriolar abnormalities were substantially worsened in animals treated with L-NAME, although the changes did not attain statistical significance. On the other hand, L-Arg treatment significantly improved ($P < 0.05$) the CsA-induced arteriolopathy. In addition, L-Arg treatment did not improve the tubulointerstitial fibrosis and arteriolopathy to the level observed in the kidneys of VH-treated animals.

Expression of TGF-β1 and PAI-1

Transforming growth factor-β1 mRNA was progressively increased in the CsA group by 7 days ($P < 0.05$) and significantly more so at 28 days ($P < 0.001$) when compared with the VH group (Figs. 2 and 3). L-NAME or L-Arg treatment in the VH groups had no effect on TGF-β1 mRNA expression. In the CsA group, treatment with L-NAME was associated with an increased TGF-β1 expression that was observed as early as 7 days ($P < 0.01$), but became more significant ($P < 0.001$) at 28 days compared with the VH group. In contrast, treatment of the CsA group with L-Arg reduced TGF-β1 expression at seven days down to the VH group level ($P < 0.001$ vs. CsA group). At 28 days, TGF-β1 expression was reduced by 33% in the CsA + L-Arg group compared with the CsA group ($P < 0.001$ vs. CsA), although L-Arg treatment was not capable of reducing TGF-β1 expression down to the VH group level.

The expression of PAI-1, a protease inhibitor that blocks matrix degradation and is directly stimulated by TGF-β, is shown in Figures 2 and 4. The expression of PAI-1 paralleled that of TGF-β1, and the changes were similar for both the mRNA expression by Northern and

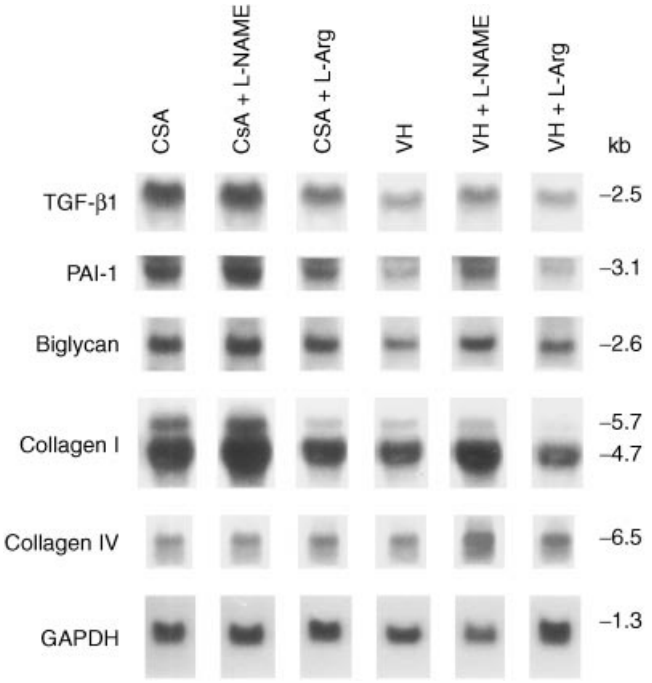

Fig. 2. Northern blots of mRNA from the cortex of salt-depleted rat kidneys. Representative gel of total RNA isolated from whole cortex at 28 days from rats treated with CsA (7.5 mg/kg/day), CsA + L-NAME (3.5 mg/kg/day), CsA + L-Arg (1.7 g/kg/day), VH, VH + L-NAME, and VH + L-Arg. RNA was hybridized with cDNA probes to TGF-β1, PAI-1, biglycan, type I collagen, type IV collagen, and GAPDH. Molecular size markers are shown on the right. Abbreviations are as follows: CsA, cyclosporine; L-NAME, N-nitro-L-arginine-methyl ester; L-Arg, L-arginine; VH, placebo.

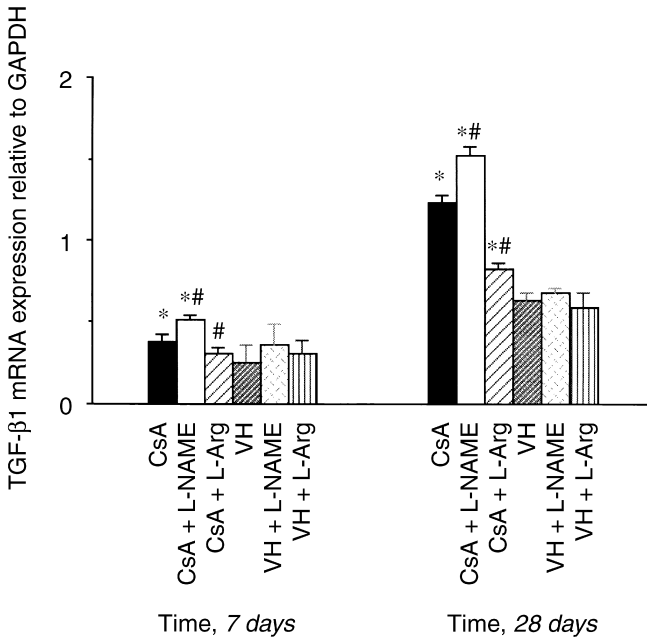

Fig. 3. Northern blot expression of transforming growth factor-β1 (TGF-β1) mRNA in rat kidneys relative to GAPDH. Total RNA was isolated from whole cortex at 7 and 28 days and was hybridized with a cDNA probe to TGF-β1. Abbreviations are as follows: CsA, cyclosporine; L-NAME, N-nitro-L-arginine-methyl ester; L-Arg, L-arginine; VH, placebo. $N = 6$ per group. *$P < 0.05$ vs. VH; #$P < 0.05$ vs. CsA.

the protein expression by ELISA. Most of the changes in the CsA groups were observed as early as 7 days ($P < 0.01$), but were more dramatic at 28 days ($P < 0.001$) when compared with the VH groups. L-NAME treatment was associated with increased PAI-1 expression at 7 and 28 days, and the changes were similar for both Northern and ELISA. In sharp contrast, treatment with L-Arg was associated with a drop in PAI-1 mRNA and protein expressions to the levels observed in the VH-treated groups for both 7 and 28 days.

Extracellular matrix deposition

The proteoglycan biglycan is an extracellular matrix component that is directly stimulated by TGF-β; its mRNA expression is shown in Figures 2 and 5. The expression of biglycan was similar to TGF-β1 and was progressively elevated in the CsA-treated group compared with the VH group at 7 days ($P < 0.05$) and 28 days ($P < 0.001$), suggesting active matrix synthesis. Biglycan mRNA expression was not affected by NO modulation in the VH groups. Concomitant treatment with L-NAME in the CsA group resulted in a significant increase in biglycan expression when compared with the CsA only ($P < 0.01$) and the VH-treated ($P < 0.01$) groups for both 7 and 28 days. On the other hand, L-Arg treatment significantly lowered the biglycan mRNA expression in the CsA group at 7 and 28 days to the levels observed in the VH groups ($P < 0.001$).

The protein expression of the glycoprotein fibronectin was studied by ELISA and is shown in Figure 6. CsA treatment did not affect fibronectin expression until 28 days, when it became significantly higher than the VH group ($P < 0.01$). The CsA + L-NAME group resulted in a markedly increased fibronectin expression that was seen at both 7 days ($P < 0.01$) and 28 days ($P < 0.001$). Concomitant therapy with L-Arg in the CsA rats brought the fibronectin levels down to that in the VH groups as early as 7 days and was persistent at 28 days. The expression of fibronectin was similar in the VH, VH + L-NAME, and VH + L-Arg groups.

Collagen type I is normally present in the interstitial component of the kidney, and its expression is increased in pathological conditions under TGF-β influence. The mRNA expression of collagen type I is shown in Figures 2 and 7A. In the CsA group, type I collagen mRNA was similar to TGF-β1 and PAI-1 and was up-regulated throughout the study period ($P < 0.01$) relative to the VH group. Concomitant treatment with L-NAME resulted in an increase in the expression of collagen type I relative to the CsA group as early as 7 days ($P < 0.05$), although a more significant increase was seen at 28 days

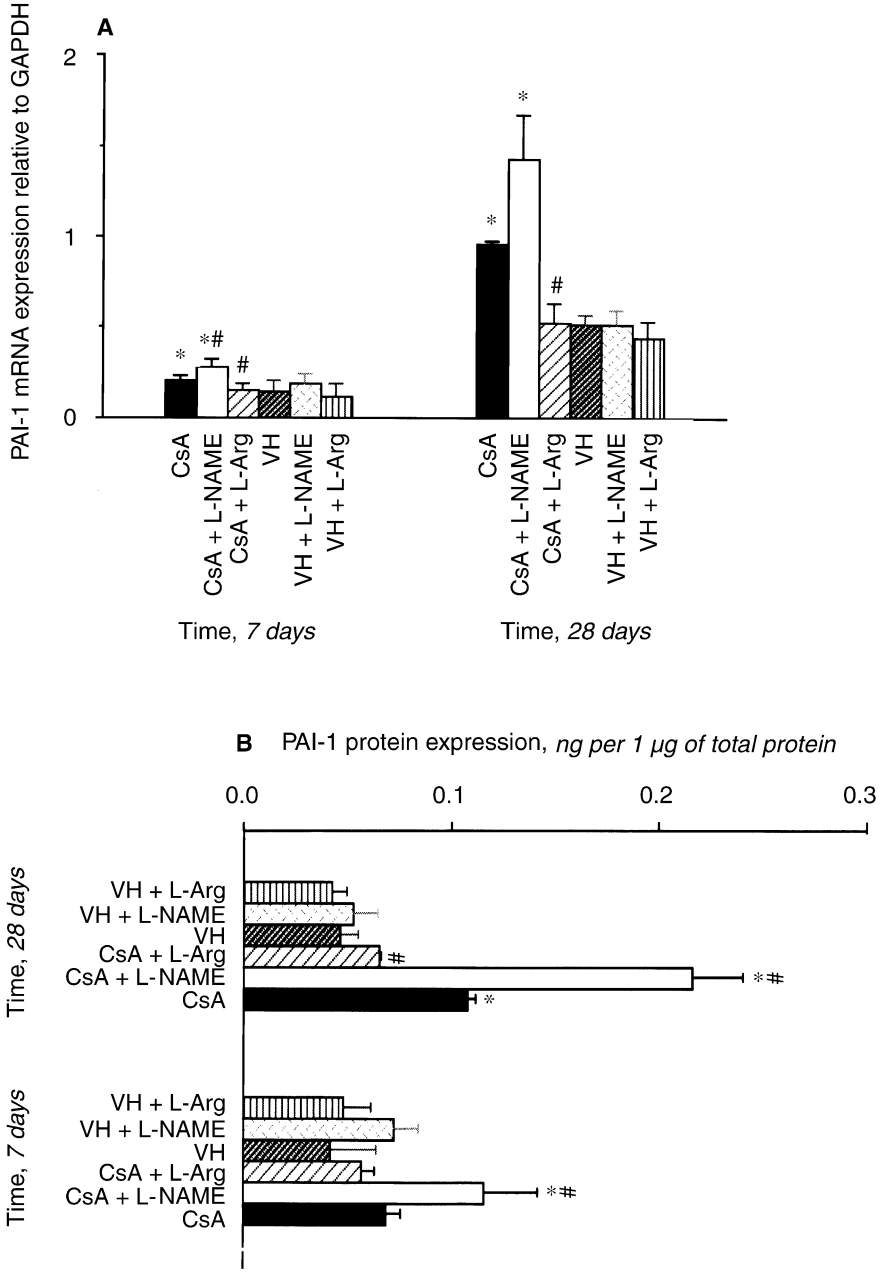

Fig. 4. Expression of PAI-1 in CsA and VH treated rat kidneys. (*A*) Total RNA was isolated from whole cortex at 7 and 28 days and was hybridized with a cDNA probe to PAI-1. (*B*) PAI-1 protein expression was determined by ELISA from whole cortex homogenates. Abbreviations are in the legend to Figure 3 ($N = 6$ per group). *$P < 0.05$ vs. VH; #$P < 0.05$ vs. CsA.

($P < 0.001$). Treatment with L-Arg was successful in normalizing the collagen type I mRNA levels to the VH group's level. No effect relative to collagen type I expression was seen in the six VH-treated groups. On the other hand, the mRNA expression of collagen type IV (shown in Figs. 2 and 7B) was similar in all the treatment groups, indicating that the effect of CsA on collagen synthesis was specific to collagen type I.

DISCUSSION

The vasoconstricting effect of CsA on the renal vasculature clearly has been shown to be involved in acute CsA nephrotoxicity, but has also been hypothesized to be important in causing renal dysfunction and ultimately renal fibrosis [35]. Several studies have suggested that endothelial-derived NO may be involved in the hemodynamic alterations encountered with CsA treatment [24, 36]. CsA-induced acute renal dysfunction was shown to improve by the simultaneous administration of L-Arg in rats [18], while NO blockade with L-NAME enhanced acute CsA nephrotoxicity [25]. NO modulation was also shown to be involved in ameliorating or worsening the effect of other nephrotoxic drugs, perhaps indicating a nonspecific effect on the kidney. However, most of the

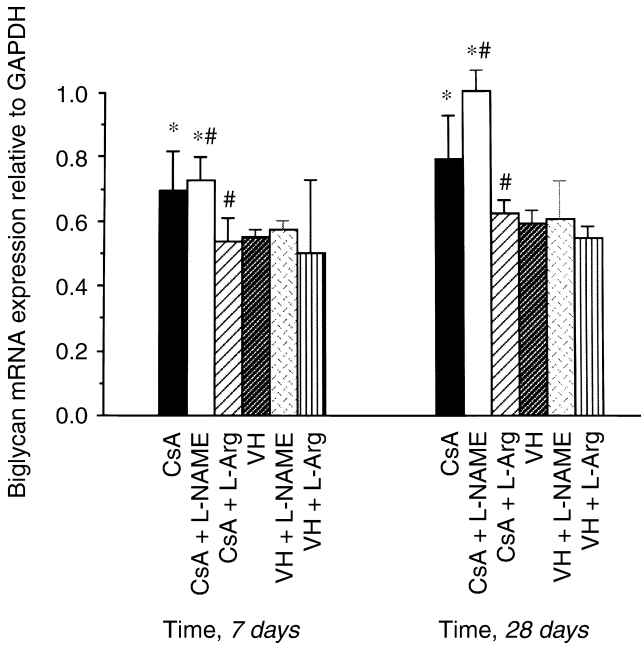

Fig. 5. Northern blot expression of biglycan mRNA in rat kidneys relative to GAPDH. Total RNA was isolated from whole cortex at 7 and 28 days and was hybridized with a cDNA probe to biglycan. Abbreviations are in the legend to Figure 3 ($N = 6$ per group). *$P <$ 0.05 vs. VH; #$P < 0.05$ vs. CsA.

studies investigating the role of the L-Arg/NO pathway have looked at the short-term administration of CsA. More recently, L-Arg administration was shown to be beneficial in experimental chronic CsA nephrotoxicity by limiting tubulointerstitial fibrosis and tubular atrophy [26]. However, the exact molecular mechanism underlying this beneficial effect has not yet been determined.

The current data confirm that chronic NO blockade with L-NAME worsens and chronic NO enhancement with L-Arg attenuates chronic CsA nephrotoxicity. Both the functional and structural manifestations of chronic CsA nephrotoxicity were affected. We have previously shown an elevated expression of TGF-β1, PAI-1, and certain matrix components in this model of chronic CsA nephrotoxicity [5–7]. In the current experiment, inhibiting NO production with L-NAME was associated with an increased TGF-β1 expression, which in turn was associated with an increase in the accumulation of matrix components; PAI-1 expression was also increased. On the other hand, therapy with L-Arg was associated with a decrease in TGF-β1, PAI-1, and matrix proteins. These results are similar to the observation in a model of obstructive nephropathy in which NO generation ameliorated tubulointerstitial fibrosis, although the expression of TGF-β1 mRNA was not affected [37]. In our experiment, this effect seems to be specific since type IV collagen expression was not influenced by NO modulation. In addition, the impact of NO modulation on TGF-β1 and matrix protein expression was not observed in the VH groups, ruling out a nonspecific effect and suggesting a specific modulatory effect on CsA. We then proposed that NO modulation affected fibrosis by altering TGF-β1 expression, which then resulted in increased matrix deposition and decreased matrix degradation through increased PAI-1.

Our study also showed that urinary NO excretion is enhanced with L-Arg treatment and blocked by L-NAME. We are aware of a recent study that suggested that urinary nitrite plus nitrate measurements may not accurately reflect acute NO generation [38]. However, our results are in agreement with other findings in the remnant kidney model of chronic renal failure [39] and in rats with unilateral kidney obstruction [37].

In addition to its effect on glomerular hemodynamics, NO plays a direct role in modulating fibrosis [40]. Indeed, in vitro and in vivo studies suggest that iNOS-generated NO may be antifibrotic and therefore may serve to limit the severity of glomerular and interstitial fibrosis [41]. Rats with unilateral ureteral obstruction treated with L-Arg showed a reduced expression of collagen type IV, α-smooth muscle actin, and tissue-inhibitor of metalloproteinase-1 mRNA [37]. In another study, the addition of NO-generating compounds to vascular smooth muscle cells [42] or mesangial cells [16] in culture significantly decreased collagen synthesis, although the exact mechanism of this beneficial effect was not determined. Similar findings were observed in the remnant kidney model in which L-Arg administration reduced proteinuria and decreased the number of abnormal glomeruli and the severity of the tubulointerstitial changes [11]. In addition, diabetic rats given L-Arg had a significantly lower protein excretion; L-Arg also prevented the development of hyperfiltration despite persistent hyperglycemia [12]. The role of NO in glomerular diseases, however, remains controversial since it provides protection in some models while it has a detrimental effect in other models of glomerulonephritis [10, 40, 41, 43]. In this article, we found significant reductions in the expression of various matrix components, including proteoglycans, biglycans, and collagens.

It was postulated that some of the beneficial effects of NO on decreasing fibrosis are related to decreasing the amount of macrophage infiltration, as shown in obstructive nephropathy, in puromycin-induced nephrosis, and in the remnant kidney model [17, 37, 39, 41]. Similarly, our CsA model displays features of interstitial inflammation, including a mononuclear infiltrate. This macrophage influx occurs early and precedes interstitial fibrosis [44]. It was also observed when low CsA doses were used in the setting of preserved renal blood flow and preceded increases in arteriolar TGF-β expression [45]. Although the degree of this mononuclear infiltrate was not quantitated in this study, we have noted a de-

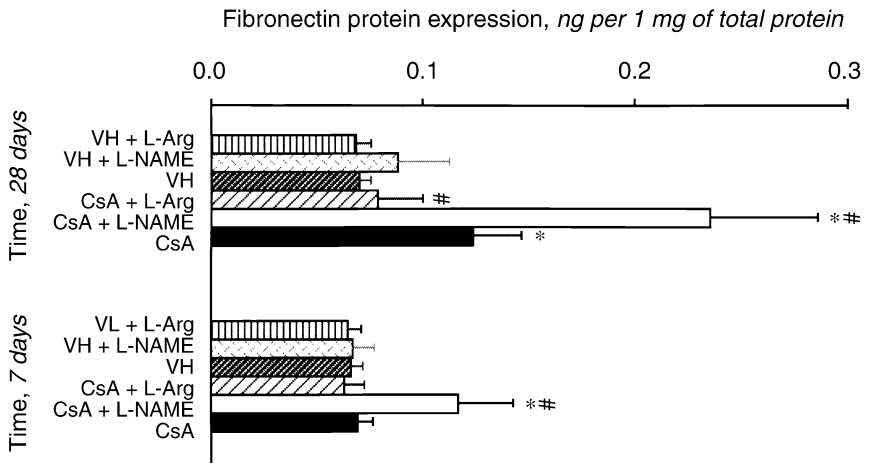

Fig. 6. Fibronectin protein expression by ELISA in rat kidneys. PAI-1 protein expression was determined by ELISA from whole cortex homogenates. Abbreviations are in the legend to Figure 3 ($N = 6$ per group). *$P <$ 0.05 vs. VH; #$P < 0.05$ vs. CsA.

crease in the amount of this infiltrate with L-Arg treatment (unpublished observation), suggesting that it may also be a contributory factor. In addition to the previously mentioned, one can speculate that the NO protective effect may also be related to its ability to inhibit leukocyte recruitment through a P-selectin mechanism [46], to inhibit adhesion molecules [47], and to inhibit the aggregation of platelets [48], a major source of TGF-β production.

There are many reports that implicate angiotensin II (Ang II) as a trigger for NO production. Studies using afferent arteriolar vessel preparations show that NOS blockade is associated with increased response to Ang II, as indexed by larger and prolonged reduction of arteriolar lumen in vitro [49]. On the other hand, Ang II receptor blockers or angiotensin-converting enzyme (ACE) inhibitors prevent most of the changes in the renal and glomerular hemodynamics induced by NOS blockade [50, 51]. More specifically, the CsA-mediated inhibition of NO synthesis resulted in the activation of intrarenal Ang II and abolished glycine-mediated vasodilation; however, pretreatment with an Ang II antagonist normalized the NO blockade effects [52]. It is also recognized that ACE inhibitors increase NO generation within the endothelium of tissues [53]. In addition, ACE inhibitors increase kinin levels, which indirectly increase endothelial NO formation [54]. These evidence suggest that NO plays a role in antagonizing Ang-II induced vasoconstriction in the kidney.

Angiotensin II plays an important role in the progression of glomerular and tubulointerstitial diseases. It stimulates extracellular matrix protein synthesis, thereby opposing the effect of NO. Ang II exhibits these actions at least partly through an induction of TGF-β [55]. We have previously shown that the RAS is up-regulated in experimental chronic CsA nephrotoxicity [5, 6], and that the expression of TGF-β1 and extracellular matrix protein synthesis was blunted by an ACE inhibitor or an Ang II receptor blocker [7]. Ang II and TGF-β are known to inhibit cytokine-induced NO production in vascular smooth muscle cells, macrophages, astroglial cells, and recently, mesangial cells [56–59]. Ang II was shown to decrease NO production in these cell lines by inhibiting *NOS-II* gene expression. TGF-β was shown to decrease NO production in macrophages by decreasing the stability and translation of NOS-II mRNA and by increasing the degradation of NOS-II protein [57]. In mesangial cells, TGF-β markedly decreases the half-life and steady-state levels of NOS-II mRNA [59]. No matter what the mechanism of action, inhibition of NO production might be one of the mechanisms by which Ang II and TGF-β accelerate fibrosis in renal diseases. This, in turn, may partly explain the beneficial effect on fibrosis with Ang II blockade or with NO supplementation in chronic CsA nephrotoxicity. However, similar to Ang II blockade [7], NO supplementation in this experiment did not totally prevent fibrosis, suggesting that a number of mechanisms may be involved in the development of chronic CsA nephrotoxicity. In addition, work with chronic models of fibrosis suggests that, after a point, further tissue injury may not be required to sustain TGF-β overexpression, indicating that there are components of the fibrotic process that can become self-perpetuating.

In addition, recent in vitro studies of glomerular mesangial cells exposed to interleukin-1 revealed that cells expressing iNOS did not undergo apoptosis, whereas cells not expressing iNOS died [60]. While apoptosis is beneficial in some disease processes, it can be deleterious at times; apoptosis may result in tissue disruption if enough cells are lost [61]. In this model, we have previously shown that CsA favors apoptosis as evidenced by an up-regulated expression of p53, Bax, and Fas ligand and a decreased expression of Bcl-2. In addition, the caspase system is activated [62]. If apoptosis is favored by NO inhibition, then a state of NO deficiency induced

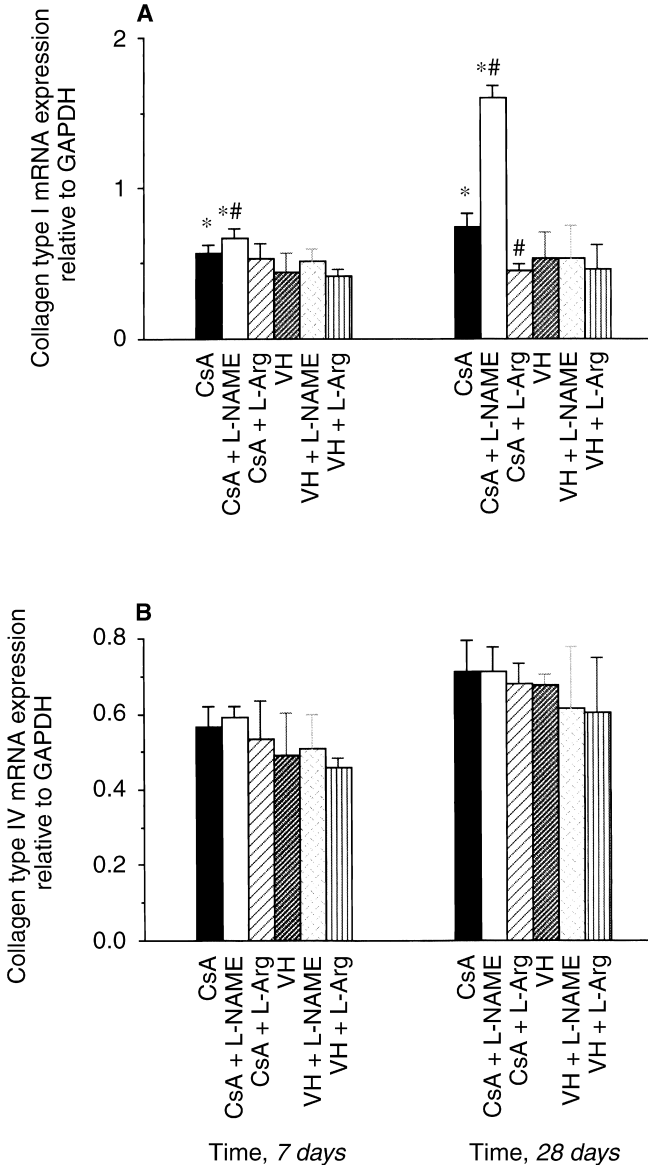

Fig. 7. Northern blot expression of collagen type I and type IV mRNA in rat kidneys relative to GAPDH. Total RNA was isolated from whole cortex at 7 and 28 days and was hybridized with a cDNA probe to collagen type I (*A*) or type IV (*B*). Abbreviations are in the legend to Figure 3 ($N = 6$ per group). *$P < 0.05$ vs. VH; #$P < 0.05$ vs. CsA.

by CsA could partly explain the increased apoptosis observed in this model.

In summary, we have provided evidence that L-Arg treatment significantly enhanced urinary NO biosynthesis and protected animals from impaired GFR and from the development of tubulointerstitial fibrosis, whereas, in contrast, L-NAME strikingly worsened chronic CsA nephrotoxicity. These effects were not dependent on alterations of systemic blood pressure. NO modulation affected fibrosis by altering TGF-β1 expression, which, in turn, was associated with increased matrix deposition and decreased matrix degradation through its effect on PAI-1. Since TGF-β is implicated in CsA-induced immunosuppression, it is still unclear how NO modulation, by affecting TGF-β expression, would impact the immunosuppressive activities of CsA. Whether L-Arg supplementation will be beneficial in humans treated with CsA remains unclear. The few human studies evaluating L-Arg supplementation in CsA therapy have not been encouraging. In one study, CsA-treated liver and renal transplant patients showed no improvement in renal function with short-term intravenous infusion of L-Arg [63]. In addition, L-Arg infusion in renal allograft recipients receiving CsA did not result in improvement in GFR or renal vascular resistance [64]. However, those studies looked at the effect of the short-term administration of L-Arg. It remains unknown as to whether long-term L-Arg supplementation will be beneficial in preventing or reducing the renal fibrosis observed with chronic CsA therapy.

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